The EMBO Journal vol.15 no.16 pp.4392–4401, 1996

Translational regulation of human p53 gene expression

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In blast cells obtained from patients with acute myelogenous leukemia, p53 mRNA was present in all the samples examined while the expression of p53 protein was variable from patient to patient. Mutations in the p53 gene are infrequent in this disease and, hence, variable protein expression in the majority of the samples cannot be accounted for by mutation. In this study, we examined the regulation of p53 gene expression in human leukemic blasts and characterized the p53 transcripts in these cells. We found control both at the level of RNA abundance and at the level of translation. Four experiments point towards translational control of human p53 gene expression. First, there is no correlation between the level of p53 mRNA and the level of p53 protein expression in blast cells. Second, in two cell lines with similar levels of p53 protein expression but with different levels of p53 mRNA, we find that there is preferential association of p53 mRNA with large polysomes in the cells with less p53 RNA. Third, translation of synthetic human p53 transcripts in cell-free extracts is inhibited by the p53 3′UTR. Fourth, the p53 3′UTR, when present *in cis*, can repress translation of a heterologous transcript. These observations raise the possibility that human p53 mRNA translation may be regulated *in vivo* by RNA binding factors acting on the p53 3′UTR.

Keywords: acute myelogenous leukemia/p53/translational control

Introduction

Human acute myelogenous leukemia (AML) is a clonal disease arising in a very early hematopoietic progenitor cell following multiple carcinogenic events (Wiggans *et al.*, 1978; Fialkow *et al.*, 1987). Mutation of the p53 tumor suppressor gene occurs infrequently in the blast cells of AML patients (Fenaux *et al.*, 1991, 1992; Slingerland *et al.*, 1991; Sugimoto *et al.*, 1991, 1993; Zhang *et al.*, 1992; Trecca *et al.*, 1994; Wattel *et al.*, 1994; Lai *et al.*, 1995). p53 mutations have been detected in ~10% of all AML patients, mostly in patients with 17p monosomy who had lost the normal remaining p53 allele (Lai *et al.*, 1995). These studies demonstrate that p53 mutations are not required for the development of AML. Mutations that do arise, however, are generally recessive in nature, indicating a strong selective pressure to eliminate completely wild-type p53 protein function.

The scarcity of p53 gene mutations in AML is not unique to this disease. For example, p53 gene mutations are rare in neuroblastoma, testicular tumors and HPV-positive cervical cancer. While the p53 gene is most commonly inactivated through mutation in human tumors, p53 protein function can also be disrupted through non-genetic mechanisms including protein–protein interactions (Scheffner *et al.*, 1990; Momand *et al.*, 1992; Oliner *et al.*, 1992; Ueda *et al.*, 1995), protein conformational change (Milner, 1991; Ullrich *et al.*, 1992) and nuclear exclusion (Moll *et al.*, 1992, 1995). Indeed, two groups have suggested that inactivation of wild-type p53 protein in AML occurs through a mechanism involving conformational change of the protein (Zhu *et al.*, 1993; Zhang *et al.*, 1992).

The level of p53 protein expression in primary blast cells obtained from AML patients varies from patient to patient. In previous studies from this laboratory p53 protein expression was detected in only 45% (34 of 75) blast samples examined by metabolic labelling with [^{35}S]methionine and immunoprecipitation (Smith *et al.*, 1986; Benchimol *et al.*, 1989; Slingerland *et al.*, 1991). Zhang *et al.* (1992) detected p53 protein expression in blast samples from 75% (37 of 49) AML patients. Several reasons may explain the absence or very low level of p53 protein expression in certain blast samples. These include low levels of p53 mRNA, inhibition of p53 mRNA translation and extremely rapid turnover of newly synthesized p53 protein. In this study, we have examined the regulation of p53 gene expression in human AML blasts and find control both at the level of RNA abundance and at the level of translation. Translational regulation is supported by experiments in which we demonstrate that the p53 3′ untranslated region (3′UTR) can repress translation of p53 RNA and of heterologous transcripts in cell-free extracts.

Results

Expression of p53 protein in human AML

Leukemic blast cells from AML patients and three human acute leukemia cell lines OCI-M2, OCI/AML-3 and OCI/AML-4 were characterized for p53 protein expression by metabolic labelling and immunoprecipitation. OCI-M2 is a human erythroleukemia cell line (Papayannopoulou *et al.*, 1988) previously shown to contain a missense mutation in the p53 coding region at codon 274 and to have lost the homologous wild-type p53 allele (Slingerland *et al.*, 1991). OCI/AML-3 and OCI/AML-4 cell lines were derived from the primary blasts of two AML patients (Wang *et al.*, 1989). The full-length p53 transcripts in these cells were amplified by RT–PCR, and the products directly sequenced. We found that the p53 transcripts in both cell lines were wild-type throughout their coding

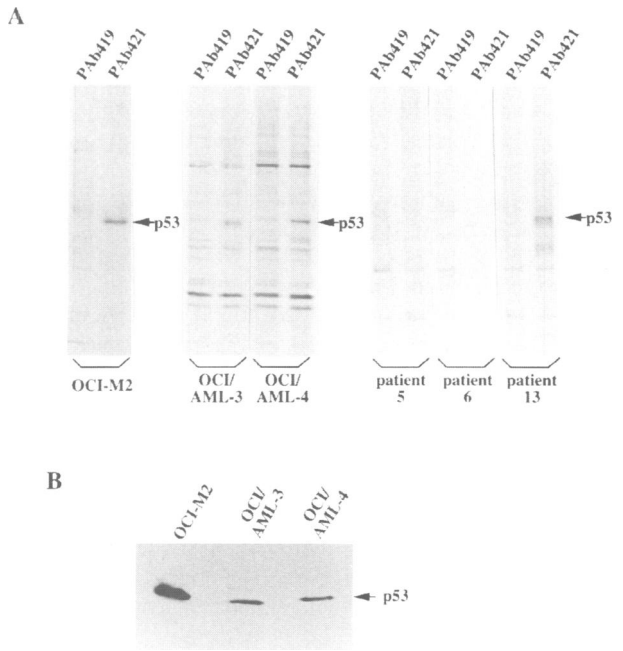

Fig. 1. Expression of p53 protein in human leukemia cells. (**A**) Cell lines OCI-M2, OCI/AML-3 and OCI/AML-4, and blast cells from AML patients were metabolically labelled with [^{35}S]methionine for 15 min at 37°C. Cell extracts were prepared and portions representing equal amounts of trichloroacetic acid-insoluble radioactivity (10^7 c.p.m.) were immunoprecipitated with the control monoclonal antibody (PAb419) or with monoclonal antibodies against p53 (PAb421). (**B**) Detection of p53 protein in 5×10^6 cells by Western immunoblotting and ECL using PAb1801 monoclonal antibodies.

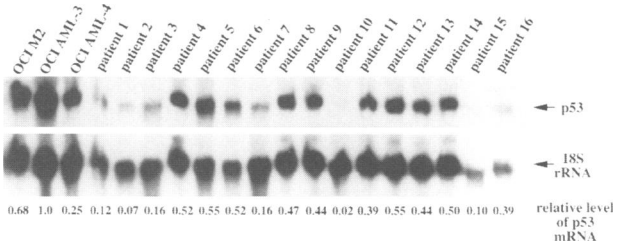

Fig. 2. Northern blot analysis of p53 mRNA in human AML cells. 20 μg of total RNA isolated from cell lines or patient blast samples was separated on a 1% agarose gel containing 6% formaldehyde, transferred to nitrocellulose and hybridized with ^{32}P-labelled human p53 cDNA. After autoradiography, the probe was removed and the filters were hybridized with a probe specific for 18S ribosomal RNA. The relative abundance of p53 mRNA was determined by phosphorimage analysis after normalizing to the value of 18S ribosomal RNA in each sample.

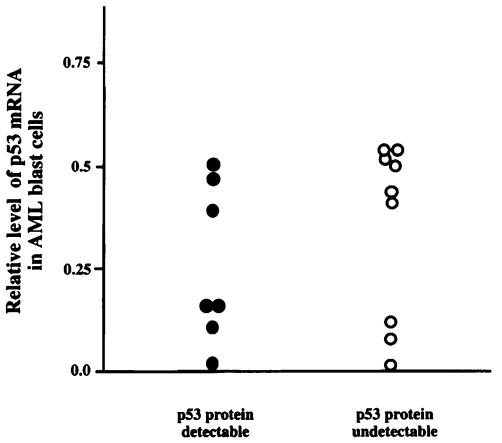

Fig. 3. Relative abundance of p53 mRNA in cells that do or do not express detectable p53 protein. p53 protein synthesis was assessed in 16 AML blast samples by metabolic labelling with [^{35}S]methionine for 15 min and immunoprecipitation. p53 protein synthesis was detected in seven of these samples. p53 mRNA levels were determined by Northern blot analysis as described in the legend to Figure 2.

regions as well as through their 5′- and 3′UTRs. The only difference detected in the p53 transcripts expressed in OCI/AML-3 and OCI/AML-4 cell lines was the recognized polymorphism at codon 72 (Matlashewski *et al.*, 1987) resulting in an arginine residue in OCI/AML-3 and a proline residue in OCI/AML-4 at position 72.

The level of protein expression measured by metabolic labelling and immunoprecipitation is dependent primarily on the rate of protein synthesis, the rate of protein degradation and the amount of mRNA available for translation. To minimize the contribution of protein half-life on the detection of p53 protein synthesis during the metabolic labelling assay, cells were exposed to a short 15 min pulse of [^{35}S]methionine at 37°C followed by immediate lysis on ice in the presence of protease inhibitors. Radiolabelled cell extracts prepared in this way were then subjected to immunoprecipitation with p53-specific antibodies. p53 protein with a half-life much less than 15 min, however, might remain undetectable by this assay. p53 protein synthesis was detected in OCI/AML-3, OCI/AML-4 and in OCI-M2 (Figure 1A) as well as in seven of 16 blast samples tested; three representative examples are shown in Figure 1A.

The steady-state level of p53 protein in the three cell lines was determined by Western blot analysis using PAb1801. Densitometric scanning of the blot shown in Figure 1B revealed that the amount of p53 protein in OCI/AML-3 and OCI/AML-4 was similar and ~10-fold lower than in OCI-M2. The high level of p53 protein in OCI-M2 was expected since mutant p53 polypeptides usually have much longer half-lives than wild-type p53 proteins and as a result mutant p53 polypeptides accumulate intracellularly.

Expression of p53 mRNA in human AML

To determine whether the differences in p53 protein expression in leukemic blasts reflected differences in the abundance of p53 mRNA, RNA was isolated from AML blast samples and cell lines, and subjected to Northern blot analysis. The relative abundance of p53 mRNA in cells was estimated by phosphorimage analysis after normalizing to the value of 18S ribosomal RNA in each sample. The results are shown in Figure 2 and indicate that the 16 AML blast samples examined synthesized a single species of full-length p53 mRNA ~2.8 kb in size. The relative amount of p53 mRNA in the 16 samples varied over a 27-fold range. No correlation was evident between p53 protein expression (on the basis of the 15-min metabolic labelling assay) and the level of p53 mRNA in AML blasts (Figure 3).

OCI/AML-3 and OCI/AML-4 cells contained similar amounts of p53 protein. However, the RNA blot shown in Figure 2 indicated that the abundance of p53 mRNA was 4-fold higher in OCI/AML-3 than in OCI/AML-4. A 4- to 8-fold difference in p53 RNA was seen in repeated

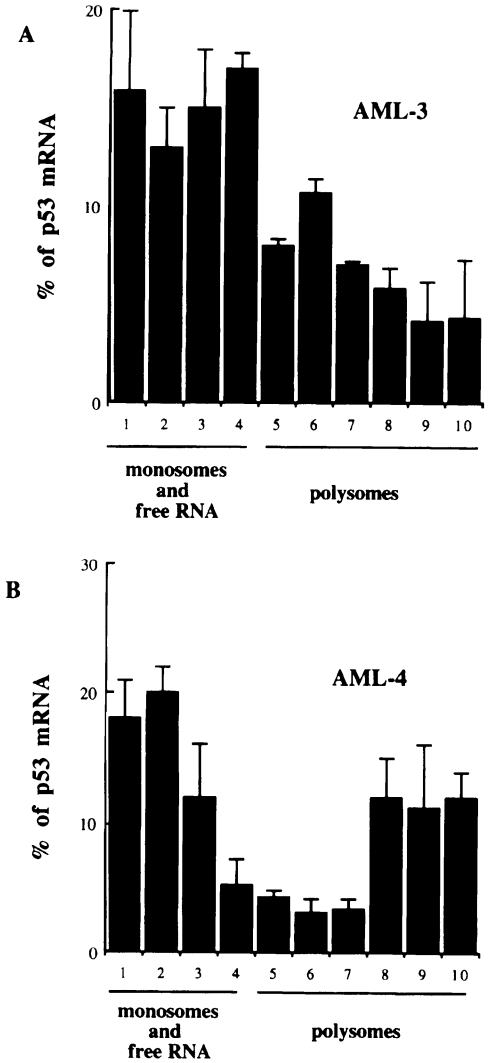

Fig. 4. Association of p53 mRNA with polysomes in OCI/AML-3 (**A**) and OCI/AML-4 (**B**) cells. The association of p53 mRNA with polysomes in OCI/AML-3 and OCI/AML-4 cells was compared. Cell extracts containing polysomes were prepared in the presence of cycloheximide and loaded on a 15–50% linear sucrose gradient. Ten fractions were collected and the amount of p53 mRNA in each fraction was determined by dot-blot hybridization analysis with a ^{32}P-labelled human p53 cDNA probe. The size of the polysomes with respect to the gradient was estimated using a polysome preparation from OCI/AML-3 cells. The positions of free ribosomes, monosomes and polysomes are indicated. Error bars represent the standard error of the mean from three separate experiments.

experiments after normalization with probes that detect 18S ribosomal RNA or GAPDH to ensure equivalent loading of RNA samples on the gels. We conclude that p53 RNA levels and p53 protein expression are variable in AML blasts and cell lines, and that the level of p53 protein expression is not related to the amount of p53 mRNA in these cells.

Association of p53 mRNA with polysomes

To test whether p53 gene expression is under translational control *in vivo*, the association of p53 mRNA with polysomes in OCI/AML-3 and OCI/AML-4 cells was analyzed (Figure 4). If p53 mRNA is more translationally active in OCI/AML-4 than in OCI/AML-3 as the above results suggest, then a larger proportion of the p53 mRNA present in OCI/AML-4 should be associated with larger polysomes compared with OCI/AML-3. Cells were collected and lysed in the presence of cycloheximide and $MgCl_2$, which stabilize the association of ribosomes with mRNA. The lysates were sedimented through a linear sucrose gradient and fractions were collected. RNA was extracted from each fraction and analyzed for the presence of p53 mRNA by dot-blot hybridization with a ^{32}P-labelled p53 cDNA probe. The gradients were calibrated with polysomes prepared from lysates by precipitation with 100 mM $MgCl_2$. Polysomes were found at the bottom of the gradient in fractions 5–10, while monosomes were found in fractions 1–4. p53 mRNA from OCI/AML-4 cells was associated with larger polysomes than was p53 mRNA from OCI/AML-3 cells (Figure 4). In OCI/AML-4 cells, 39% of the p53 mRNA was found in fractions 7–10 containing high molecular weight polysomes, while in OCI/AML-3 cells 21% of the p53 mRNA was found in these same fractions. As an internal control, the distribution of ribosomal protein L35 RNA was compared and shown to be identical in OCI/AML-3 and OCI/AML-4 (data not shown).

Analysis of the 5′ end of p53 mRNA

The human p53 gene has been shown to have a cluster of six or seven major transcription initiation sites and several minor sites lying further upstream (Tuck and Crawford, 1989). Transcripts initiating from the minor sites would have a longer 5′UTR with potential to form a stable stem–loop structure close to the 5′ cap. Such structures would not be expected to form in transcripts initiating from the major start sites. 5′-stem–loop structures were described for rodent p53 mRNA (Bienz *et al.*, 1984; Bienz-Tadmor *et al.*, 1985). Recently, mouse p53 protein was shown to bind to the 5′UTR and to inhibit translation of its own mRNA in an *in vitro* assay system (Mosner *et al.*, 1995). Stable stem–loop structures in the 5′UTR regions of a number of mRNA transcripts have been shown to inhibit translation initiation by interfering with the activity of translation initiation factors or by serving as binding sites for regulatory proteins that inhibit translation (Feng and Holland, 1988; Fu *et al.*, 1991; Melefors and Hentze, 1993; Pause *et al.*, 1993).

To determine if the low level of p53 protein expression in leukemic blasts was the result of transcription initiating at the minor start sites, the 5′ ends of p53 mRNA present in different blast samples and cell lines were mapped using an RNase protection assay. A 729 nucleotide antisense RNA probe containing genomic sequences from the p53 promoter region fused with cDNA sequences extending into exon 4 was generated by transcription with SP6 RNA polymerase in the presence of [^{32}P]UTP (Figure 5A). This probe would yield protected p53 fragments of 385 nucleotides corresponding to transcripts originating from the major start site and 449 nucleotides corresponding to transcripts originating from the most 5′ of the minor start sites. Total RNA extracted from OCI/AML-3 and OCI/AML-4 cell lines and from seven AML blast samples was examined. After digestion, the protected fragments were resolved by electrophoresis on a denaturing polyacrylamide gel. As shown in Figure 5B, the predominant protected fragment in all the RNA samples was 385 nucleotides in length indicating a common site for initiation

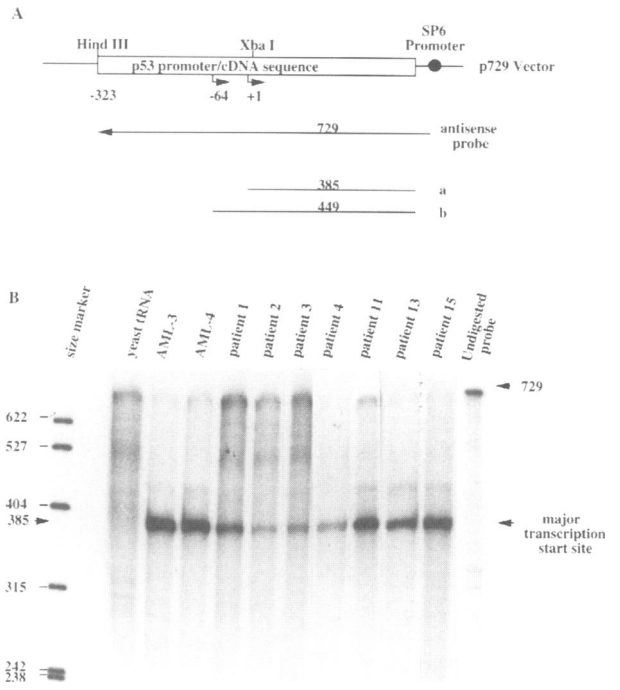

Fig. 5. RNase protection assay. (**A**) The map of the p729 plasmid. The p729 plasmid was constructed as described under Materials and methods. After linearization with *Hin*dIII, a 729 nucleotide antisense RNA probe was generated by transcription with SP6 RNA polymerase yielding protected p53 fragments of ~385 nucleotides due to p53 transcripts initiating from one of the major start sites (a) and 449 nucleotides due to p53 transcripts initiating from the most 5′ of the minor transcription start sites (b). (**B**) The 729 nucleotide [^{32}P]UTP-labelled antisense RNA probe was annealed to 30 μg of total RNA extracted from OCI/AML-3 and OCI/AML-4 cell lines and seven AML blast samples before digestion with RNase A and RNase T1. The protected fragments were separated by electrophoresis on a 6% polyacrylamide–8 M urea gel and visualized by autoradiography. The positions and size (nucleotide length) of 5′ end-labelled fragments of *Msp*I-digested pBR322 plasmid DNA are indicated on the left. The bottom arrow indicates the position of the major protected fragment and the top arrow indicates the undigested probe.

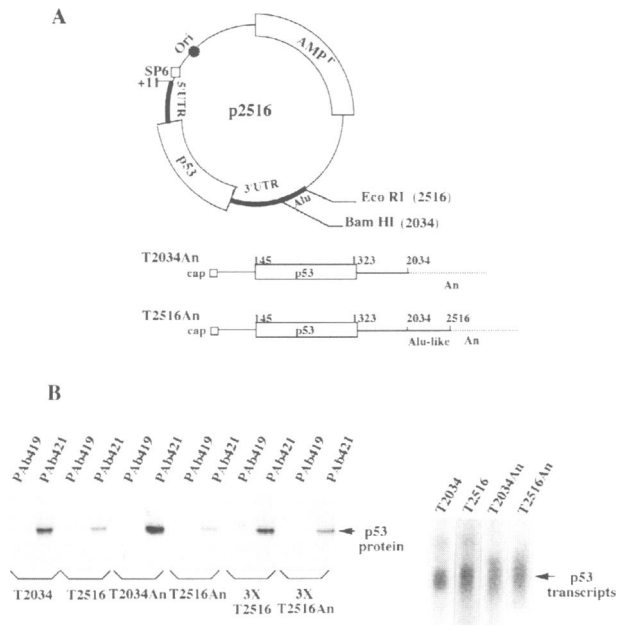

Fig. 6. *In vitro* translation of synthetic p53 RNA containing variable portions of the 3′UTR. (**A**) Plasmid template used to synthesize p53 RNA *in vitro*. The p2516 plasmid was constructed by inserting the entire 2.5 kb wild-type p53 cDNA sequence downstream of the bacteriophage SP6 promoter in a pSP64-derived plasmid. Transcription from the SP6 promoter present in p2516 leads to the production of transcripts in which the first 10 nucleotides are derived from plasmid sequences while the remaining nucleotides are derived from the p53 gene beginning at the +7 position of native p53 transcripts initiating from one of the major transcription start sites (Tuck and Crawford, 1989). Linearization of p2516 at the *Eco*RI site before *in vitro* transcription generates a full-length, Alu-containing p53 transcript (T2516); linearization at the *Bam*HI site provides a template for the synthesis of a truncated p53 transcript missing a portion of the 3′UTR containing the Alu sequence (T2034). Both transcripts were polyadenylated *in vitro* to generate p2516An and p2034An. The open rectangles shown on the transcripts represent the position of the p53 coding region. (**B**) 50 ng of the *in vitro*-synthesized T2034, T2516, T2034An and T2516An p53 RNAs were translated in a rabbit reticulocyte lysate at 30°C for 30 min in the presence of [^{35}S]methionine followed by immunoprecipitation, SDS–PAGE and autoradiography. In the 3X T2516 and 3X T2516An lanes, 150 ng of T2516 or T2516An RNA was added to the *in vitro* translation reaction. The right panel presents the results of a Northern blot in which 50 ng of synthetic p53 RNA was applied to an agarose–formaldehyde gel, blotted and hybridized to ^{32}P-labelled human p53 cDNA.

of p53 gene transcription in leukemic blasts at the major start site. These data indicate that, in contrast with murine p53 mRNA, stable secondary structures are unlikely to exist at the 5′ end of human p53 mRNA.

Analysis of the 3′ end of p53 mRNA

Human p53 mRNA contains a long 3′UTR of 1176 nucleotides with an Alu-like repetitive sequence element of ~470 bp located immediately upstream of the poly(A) tail (Matlashewski *et al.*, 1984). The Alu-like sequence is in the reverse transcriptional orientation with respect to the p53 gene. Furthermore, the Alu-like sequence is missing in murine p53 transcripts and it interrupts a region in human p53 mRNA which shows homology to mouse p53 mRNA. When analyzed with the FOLD program of GCG, the Alu-like element in the 3′UTR of human p53 mRNA is predicted to form an independent secondary structure that does not have long-range interactions with other regions of p53 mRNA. In the presence of a poly(A) tail, the secondary structure formed by the Alu-like element is predicted to remain essentially intact except that a 50 nucleotide U-rich sequence at the 5′ boundary of the Alu-like sequence will interact with the poly(A) tail. The extended base pairing between U and A residues will further stabilize the secondary structure formed by the Alu-like element. To determine whether or not the Alu-like repeat present in human p53 mRNA might constitute a negative regulatory element during translation, a series of *in vitro* transcription–translation experiments was performed.

An SP6-derived plasmid containing human wild-type p53 cDNA including the entire 3′UTR was constructed (p2516 in Figure 6A). p2516 was linearized with *Eco*RI or with *Bam*HI and used as a template for *in vitro* transcription. In some reactions, a poly(A) tail of 200–300 adenylic acid residues was added to synthetic p53 RNA using poly(A) polymerase. In this way, four synthetic p53 transcripts were generated: T2516An and T2516 represent full-length, Alu-containing transcripts with or without a poly(A) tail; T2034An and T2034 represent

shorter, Alu-deficient p53 transcripts with or without a poly(A) tail. These transcripts were then used as templates for translation in a rabbit reticulocyte lysate containing [^{35}S]methionine. p53 protein synthesized *in vitro* was immunoprecipitated with PAb421 monoclonal antibody and visualized by autoradiography (Figure 6B). The amount and integrity of the synthetic p53 RNAs added to the *in vitro* translation reactions was monitored by agarose gel electrophoresis and Northern blotting as shown in the right panel of Figure 6B. Densitometric tracing of the data indicated that the Alu-containing, non-polyadenylated transcript T2516 was translated ~3-fold less efficiently than the Alu-deficient, non-polyadenylated transcript T2034. In addition, the polyadenylated, Alu-containing transcript T2516An was translated ~20-fold less efficiently than the polyadenylated, Alu-deficient transcript T2034An. These data indicate that the Alu-like element present in the p53 3′UTR can inhibit p53 mRNA translation *in vitro*, even in the absence of a poly(A) tail. The predicted interaction of the poly(A) tail with the Alu-like element appears to increase further the inhibition of translation.

To test further the inhibitory activity of the p53 3′UTR, we examined the ability of the p53 3′UTR to control the translation of a heterologous RNA. The Alu-containing p53 DNA fragment extending from nucleotides 2034 to 2516 was excised from plasmid p2516 and inserted downstream of a heterologous gene (CAT gene) in an SP6-based plasmid vector to generate the plasmid pCAT-Alu (Figure 7A). *In vitro* transcription and translation revealed that non-polyadenylated CAT-Alu RNA was translated 5-fold less efficiently than non-polyadenylated CAT transcripts lacking the Alu sequence (Figure 7B). When a different region of the p53 3′UTR (nucleotides 1465–2034 in plasmid p2516) with approximately the same length as the Alu-containing fragment was inserted downstream of the CAT gene, no effect on CAT translation was observed (CAT-BS in Figure 7B). The ability of the Alu-containing segment of the p53 3′UTR to act on a heterologous transcript indicates that it likely represses translation independently of upstream sequences.

The inhibitory activity of the Alu-like element on p53 translation was likely the result of its action *in cis* and not simply due to non-specific inhibition of translation, since a 3-fold increase in the amount of Alu-containing transcript added to the reticulocyte lysate resulted in a corresponding increase in the amount of p53 protein synthesized (Figure 6B). Furthermore, when 200 ng of luciferase RNA was added to a reticulocyte lysate together with 200 ng of CAT-Alu or CAT-BS RNA, there was little difference in the amount of luciferase synthesized (Figure 7C). Similarly, when 200 ng of luciferase RNA was added to a reticulocyte lysate, either alone or mixed with 200 ng of T2034 or T2516An RNA, there was little difference in the amount of luciferase synthesized (data not shown).

To confirm that the decrease in p53 protein synthesis from Alu-containing p53 RNAs was due to translational regulation and not due to preferential RNA degradation in the reticulocyte lysate, adenylated T2034 and T2516 synthetic transcripts were added to the rabbit reticulocyte lysate under the same conditions as those used for *in vitro* translation. After incubation for 15 or 60 min, RNA was extracted from the lysate and the amount of synthetic p53 RNA present in the lysate determined by Northern blot analysis. Enhanced degradation of the Alu-containing transcript was not observed (Figure 8). We conclude that a segment of the p53 3′UTR encompassing the Alu-like element is capable of repressing translation *in vitro*.

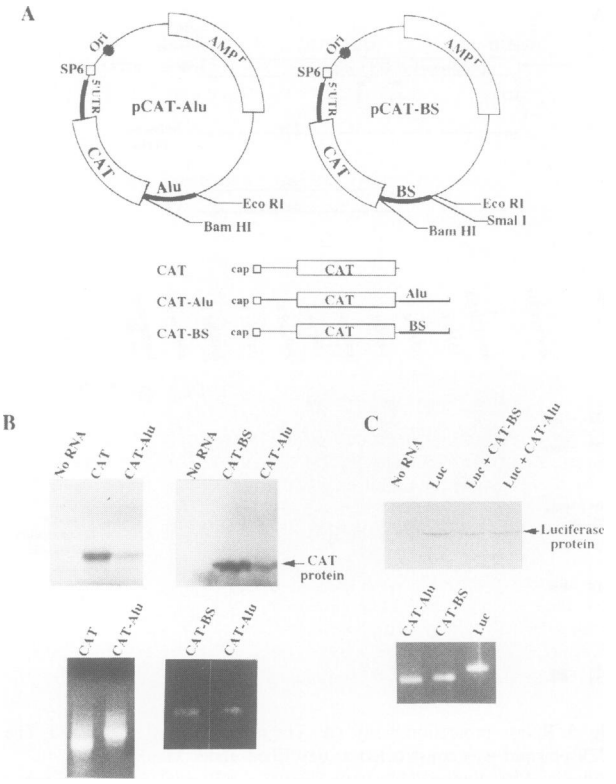

Fig. 7. The p53 Alu-like element can inhibit translation of a heterologous CAT transcript. (**A**) Plasmids used to generate CAT transcripts *in vitro*. (**B**) 200 ng of *in vitro*-synthesized CAT, CAT-Alu, and CAT-BS transcripts were translated in a rabbit reticulocyte lysate at 30°C for 30 min in the presence of [^{35}S]methionine. The reactions were stopped by adding an equal volume of the 2X protein sample buffer, heated to 100°C for 5 min and analyzed by SDS–PAGE and autoradiography. An ethidium bromide-stained agarose gel demonstrating the integrity and amount of synthetic transcripts that were added to the *in vitro* translation reaction is shown below. (**C**) 200 ng of luciferase RNA was translated in a rabbit reticulocyte lysate either alone or in the presence of 200 ng of CAT-BS or 200 ng of CAT-Alu. Reaction mixtures were incubated in the presence of [^{35}S]methionine at 30°C for 30 min and processed as in (B). The *in vitro*-synthesized luciferase protein is shown in the upper panel; the RNA used for *in vitro* translation is shown in the ethidium bromide stained-agarose gel in the bottom panel.

Discussion

The observation that wild-type p53 protein expression in leukemic blast cells does not correlate with the level of p53 mRNA mirrors findings reported previously for blasts and other human cell types (Matlashewski *et al.*, 1986; Kastan *et al.*, 1991a; Slingerland *et al.*, 1991; Sasano *et al.*, 1992; Hsu *et al.*, 1993). The absence of detectable p53 protein in cells expressing abundant levels of wild-type p53 mRNA has usually been attributed to the short half-life of p53 protein in normal cells (Rogel *et al.*, 1985). A similar situation exists in papillomavirus (HPV)-infected cells such as HeLa cells where p53 protein is not detected even though these cells produce p53 mRNA and this RNA is associated with polysomes (Matlashewski

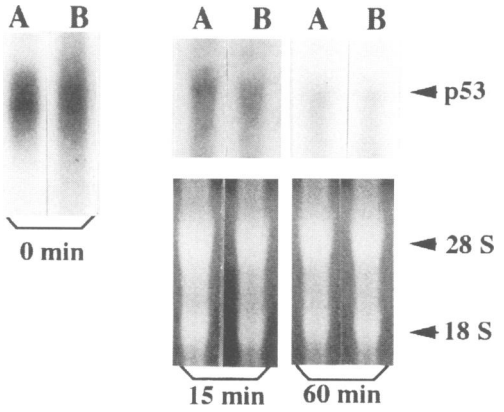

Fig. 8. Stability of synthetic human p53 RNAs in rabbit reticulocyte lysates. 100 ng of adenylated T2034 (A) and T2516 (B) synthetic RNA was added to the rabbit reticulocyte lysate and incubated at 30°C for 15 or 60 min under the same conditions used for *in vitro* translation. RNA present in the lysates was then extracted and loaded on a 1% agarose–formaldehyde gel. The 0 min time point represents 100 ng of synthetic RNA loaded directly on the gel. The amount of p53 RNA in each sample was then determined by Northern blotting using a ^{32}P-labelled human p53 cDNA. The lower panel shows the 28S and 18S ribosomal RNAs recovered from the rabbit reticulocyte lysates detected by ethidium bromide staining of the gel.

et al., 1986). The enhanced degradation of newly synthesized p53 protein in HeLa cells was shown to be promoted by the papillomavirus E6 protein which is expressed constitutively in these cells (Scheffner *et al.*, 1990).

In this report, we present data showing that differences in p53 mRNA abundance exist in AML blasts and that these differences cannot explain the heterogeneity in the level of p53 protein expression in leukemic blast cells. Using a metabolic labelling assay in which blasts from different AML patients were pulse-labelled with [^{35}S]-methionine for 15 min to minimize the contribution of protein half-life on the detection of p53 protein synthesis, we found differences in the level of p53 protein expression in blast samples. These observations raised the possibility that p53 gene expression may be regulated at the translational level in certain human cells. We tested this possibility by analyzing the distribution of p53 mRNA on polysomes *in vivo* and by examining p53 RNA translation *in vitro*.

We have used two AML cell lines, OCI/AML-3 and OCI/AML-4 that contain similar amounts of wild-type p53 protein even though OCI/AML-3 contains 4- to 8-fold more p53 mRNA. Comparison of the polysome profile of these cells indicated that a greater proportion of the p53 mRNA was associated with larger polysomes in OCI/AML-4 than in OCI/AML-3. p53 mRNA in both of these cell lines as well as in blasts from different AML patients is present as a single, full-length species of ~2.8 kb that initiates from a common transcription start site and contains similar sequence and structural elements.

Transcription–translation experiments *in vitro* indicated that the p53 3′UTR contains a negative regulatory domain that is capable of repressing translation *in vitro*. A region of the 3′UTR consisting of ~500 nucleotides and containing an Alu-like element is capable of repressing translation of p53 mRNA and of a heterologous transcript. The p53 3′UTR, when present *in cis*, repressed translation of polyadenylated as well as non-polyadenylated transcripts. Accordingly, we suggest that the Alu-like element, possibly through its secondary structure, is capable of repressing p53 mRNA translation. In addition, interaction of the Alu-like element with the poly(A) tail may repress the latter's function in translation. Experiments are in progress to map precisely this regulatory element in the p53 3′UTR and to determine if the p53 3′UTR plays a similar role in regulating translation *in vivo*.

Our finding that p53 protein expression in AML blasts is controlled, at least in part, through mechanisms acting at the translational level, raises the possibility that translational regulation may provide an epigenetic mechanism to reduce or even eliminate wild-type p53 protein function in leukemic blasts. In preliminary experiments to address this point, we have exposed blast cells that express little or no detectable p53 protein to 6 Gy of ionizing radiation and have observed increased steady-state levels of p53 protein at 1.5 h after irradiation (data not shown). Genotoxic agents have been shown previously to increase the level and/or activity of p53 protein through a post-transcriptional mechanism that is not well understood (Kastan *et al.*, 1991b; Fritsche *et al.*, 1993; Lu and Lane, 1993; Zhan *et al.*, 1993). Hence, blast cells retain the ability to up-regulate p53 expression in response to genotoxic stress. At least under these conditions, p53 function may not be lost. This type of analysis, however, does not address the function of p53 in proliferating cells that have not been exposed to genotoxic stress. In this regard, previous studies from our laboratory demonstrated a highly significant correlation between p53 protein expression in leukemic blast cells and the secondary plating efficiency of these cells (Smith *et al.*, 1986). The latter provides an estimate of the self-renewal capacity of progenitor cells in the blast population. Deregulated p53 expression might, therefore, be expected to affect the self-renewal capacity of blasts in the absence of genotoxic stress.

Accumulating evidence demonstrates the involvement of the 3′UTR in translational control (Jackson, 1993). The demonstration that the 3′UTR of certain transcripts can control mRNA localization and polyadenylation provides a mechanism for translational regulation (Huarte *et al.*, 1992; Gavis and Lehmann, 1994). In addition, specific sequences within 3′UTRs have been shown to repress translation (Goodwin *et al.*, 1993; Evans *et al.*, 1994; Kwon and Hecht, 1993). RNA–protein interactions are likely to be involved in 3′UTR-dependent translational repression. Indeed, a protein that binds specifically to the 3′UTR of protamine 2 mRNA and represses its translation has been identified (Kwon and Hecht, 1993). If the p53 3′UTR can be shown to regulate p53 mRNA translation *in vivo*, it is possible that *trans*-acting factors (missing or inactive in reticulocyte lysates) activate components of the translational machinery to bypass this negative regulatory domain on human p53 mRNA. Such *trans*-acting factors could interact directly with p53 mRNA to enhance its rate of translation. Alternatively, *trans*-acting factors directed to the p53 3′UTR (that are also present in reticulocyte lysates) may act as repressors of translation. Differences in the level of p53 protein synthesis among AML blasts and possibly other human cells could, therefore, be determined by differences in the level or activity of these regulatory molecules.

Materials and methods

Cells

The OCI/AML-3 and OCI/AML-4 cell lines were derived from primary blasts of two AML patients (Wang *et al.*, 1989). The OCI-M2 cell line was derived from the primary blasts of a patient whose erythroleukemia represented the end stage of a previously identified myelodysplastic syndrome (Papayannopoulou *et al.*, 1988). OCI/AML-3 and OCI-M2 cells were grown in alpha-modified minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) (GIBCO). The OCI/AML-4 cells were grown in α-MEM containing 10% FCS and 10% conditioned medium obtained from the human bladder carcinoma cell line 5637 (5637-CM) (Wang *et al.*, 1989). The AML blast cells were obtained directly from AML patients. The mononuclear cell fraction of peripheral blood was collected after separation through Ficoll–Hypaque (Pharmacia) (1.077 g/ml) and T-lymphocyte depletion (Minden *et al.*, 1979). These cells were stored frozen in liquid nitrogen before use.

Metabolic labelling and immunoprecipitation

The blast cells of AML patients were thawed and incubated for 2 days at 37°C in α-MEM containing 10% FCS and 10% 5637-CM before metabolic labelling. 1×10^7 cells were labelled with 0.2 mCi [^{35}S]-methionine (DuPont NEN Research Products) in 0.5 ml α-MEM lacking methionine and containing 10% dialysed FCS at 37°C for 15 min. Cells were then immediately pelleted, the radioactive medium removed, and the cells lysed on ice in a solution containing 25 mM Tris pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP40, 0.2% SDS, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin and 1 μg/ml aprotinin for 20 min. Lysates were cleared by centrifugation, the supernatant was retained and incubated with 5 μg of a non-specific IgG2a mouse monoclonal antibody (Sigma) for 60 min on ice. These were then reacted with 0.5 ml of a 10% suspension of formalin-treated *Staphylococcus aureus* Cowan 1 cells (Pansorbin, Calbiochem-Behring) for 30 min on ice, followed by centrifugation and retention of the supernatant. Portions of precleared lysates containing equal numbers of trichloroacetic acid-insoluble counts (10^7 c.p.m.) were diluted in NET/GEL buffer (150 mM NaCl, 5 mM EDTA pH 8.0, 50 mM Tris pH 7.4, 0.05% NP40, 0.02% sodium azide, 0.25% gelatin) and immunoprecipitated on ice for 2 h with PAb421 monoclonal antibodies against p53 protein or control PAb419 antibodies (Harlow *et al.*, 1981). The immune complexes were collected on 60 μl prewashed protein A–Sepharose beads (Pharmacia), washed three times with NET/GEL buffer, and eluted into 30 μl protein sample buffer (2% SDS, 10% glycerol, 0.1% bromophenol blue, 25 mM Tris pH 6.8, 0.1 M dithiothreitol) by boiling for 10 min. The Sepharose beads were removed by centrifugation, the samples were loaded on a 10% polyacrylamide gel containing SDS and proteins were resolved by electrophoresis at 45 mA. Gels were fixed in 7.5% acetic acid and 25% methanol for 30 min before drying and exposure to X-ray film (DuPont NEN Research Products).

Western blot analysis

5×10^6 cells were lysed directly in an equal volume of 2X protein sample buffer. The extracts were passed through a 21-gauge needle several times to reduce viscosity and boiled for 10 min before electrophoresis at 45 mA on a 10% polyacrylamide gel containing SDS. Resolved proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell), and the abundance of p53 protein was estimated by immunoblotting with a human p53-specific monoclonal antibody PAb1801 (Banks *et al.*, 1986). Bound antibody was detected using the enhanced chemiluminescence detection system (DuPont NEN Research Products) according to the manufacturer's instructions.

Northern blot analysis

Total cellular RNA was isolated using the guanidinium thiocyanate–cesium chloride method (Chirgwin *et al.*, 1979). 20 μg of total RNA was separated by electrophoresis on a 1% agarose gel containing 6% formaldehyde and transferred to a nitrocellulose membrane (Schleicher & Schuell). The blots were hybridized with cDNA probes labelled with [^{32}P]dCTP in a random priming reaction (Feinberg and Vogelstein, 1983), washed and exposed to X-ray film. The amount of RNA was determined with a Molecular Dynamics PhosphorImager using Multiquant software. The human p53 probe was the *Xba*I–*Eco*RI fragment of p53 cDNA from the pR4-2 plasmid (Harlow *et al.*, 1985); the L35 probe was the *Pst*I–*Bam*HI fragment from the human ribosomal protein L35 cDNA (Herzog *et al.*, 1990); the GAPDH probe was a 1.3 kb *Pst*I fragment of rat GAPDH cDNA (Fort *et al.*, 1985); the 18S ribosomal RNA probe was the *Eco*RI fragment from the human ribosomal RNA gene (Torczynski *et al.*, 1985).

Genomic DNA preparation

Genomic DNA from OCI/AML-3 and OCI/AML-4 cell lines was isolated following a modification of the procedure described by Kupiec *et al.* (1987). 3×10^7 cells were washed with ice-cold PBS buffer, resuspended in 3 ml of lysis buffer (20 mM EDTA pH 8.0, 100 μg/ml proteinase K, 0.5% sarkosyl) and incubated at 50°C for 3 h. DNA was extracted with phenol/chloroform, dialysed against 50 mM Tris–HCl pH 8.0, 10 mM EDTA, 10 mM NaCl at 4°C, and then treated with RNase A (100 μg/ml) at 37°C for 3 h. DNA was again extracted with phenol/chloroform and dialysed against 10 mM Tris pH 7.4, 1 mM EDTA. DNA concentration was determined by measuring the absorbance at 260 nm.

Amplification of p53 sequences from RNA and DNA

20 μg of total RNA was precipitated with ethanol and resuspended in a 30 μl reaction containing 300 ng of oligo(dT) primer (Amersham International), 50 mM Tris–HCl pH 8.3, 77 mM KCl, 3 mM $MgCl_2$, 3 mM dithiothreitol, 3 mM dNTP, 30 units of RNAguard (Pharmacia) and 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL) and incubated at 42°C for 60 min. The first strand cDNA was then used as the template for amplification by PCR using *Taq* polymerase (Promega). PCR amplification was performed with 10 μl of each first stand cDNA as the template and 40 cycles of denaturation (94°C, 1 min), annealing (64°C, 30 s), and elongation (72°C, 1 min). The following p53-specific primers were used for amplifying the complete coding region and the 3′UTR: 5′SX1 (sense, exon 1, GACACTTT-GCGTTCGGGCTGGGAG), 5′SX5A (sense, exon 5, GAGCGCTGCT-CAGATAGCGATG), 3′SX11 (sense, exon 11, GAAGGGCCTGACT-CAGACTGAC), 3′AX-6 (antisense, exon 6, AGATGCTGAGGAGGG-GCCAGAC), JS-3 (antisense, exon 11, GAGGGAGAGATGGGGGT-GGGAGGCTGTC) and AS-4 (antisense, exon 11, GGCAGCAAAGT-TTTATTGTAAAATAAG). The 5′UTR and sequences further upstream were amplified from 1 μg genomic DNA using the following pair of p53-specific primers: 5′UTR-1 (sense, promoter region, ACCTAA-GCTTGTCATGGCGACTGTCCAGCTTTG) and p-EX (antisense, exon 1, CCAATCCAGGGAAGCGTGTCACCG).

Direct sequencing of double-stranded PCR products

Double-stranded DNA fragments produced by PCR amplification were eluted from agarose gels and purified by extraction with phenol/chloroform. 200 ng of purified PCR product were mixed with human p53-specific oligonucleotides as sequencing primers, frozen in dry ice, dried in a centrifugal evaporator (Savant SpeedVac), redissolved in sequencing buffer (40 mM Tris–HCl pH 7.5, 25 mM $MgCl_2$, 50 mM NaCl, 10% DMSO) and subjected to the sequencing reaction as described by Winship (1989).

RNase protection assay

Plasmid p729 was constructed from three DNA fragments in two stages. A 330 bp DNA fragment derived from the human p53 gene promoter was excised from the p2E-H2BX plasmid (Lamb and Crawford, 1986) with *Hin*dIII and *Xba*I and inserted into the pGEM-4 plasmid (Promega) between the *Hin*dIII and *Xba*I sites. In the second stage, a fragment corresponding to the 5′ end of p53 mRNA was obtained by RT–PCR using p53 mRNA prepared from OCI/AML-3 cells and the p53-specific primers 5′UTR-3 (sense, exon 1, CCGGAAGCTTCAAAAGTCTA-GAGCCACCGTCCAG) and 5′AX4 (antisense, exon 4, GGTGTAGG-AGCTGCTGCTGGTGC). The resulting fragment was end-filled with the Klenow fragment of DNA polymerase I, digested with *Xba*I at the site present in the 5′UTR-3 primer shown underlined and inserted between the *Xba*I and *Sma*I sites present in the plasmid generated in the first stage.

p729 was linearized with *Hin*dIII and a 729 nucleotide antisense probe was prepared by transcription with SP6 RNA polymerase. The *in vitro* transcription reaction mixture contained 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 4 mM spermidine, 10 mM NaCl, 0.5 mM each of ATP, GTP, CTP, 12 μM UTP, 5 μCi [^{32}P]UTP, 10 mM dithiothreitol, 20 units of RNAguard, 0.5 μg of linearized template and 10 units of SP6 RNA polymerase in a final volume of 20 μl. After incubation at 37°C for 60 min, the DNA template was digested with DNase I and the RNA probe was extracted with phenol/chloroform, precipitated with ethanol and resuspended in water. This RNA probe covered the entire p53 gene promoter region and included the first three exons and a part of the fourth exon. p53 transcripts initiating from one of the major start sites

should yield protected fragments of ~385 nucleotides. p53 transcripts originating from the most 5′ of the minor start sites should yield protected fragments of 449 nucleotides (Tuck and Crawford, 1989).

In the RNase protection assay, 30 μg of total RNA was mixed with 1×10^5 c.p.m. of the labelled probe and precipitated with ethanol. The RNA/probe mixtures were then washed, dried and resuspended in 10 μl of hybridization solution (Winter *et al.*, 1985), heated to 80°C for 10 min, and hybridized at 46°C overnight. After hybridization, the samples were mixed with 0.18 ml of RNase digestion mix containing 60 μg/ml of RNase A (type III, Sigma), 1100 U/ml of RNase T1 (Boehringer Mannheim) in 300 mM NaCl, 5 mM EDTA, 10 mM Tris–HCl pH 7.5. After incubation at 37°C for 60 min, the digestion was terminated by addition of 10 μl of 20% SDS and 5 μl of proteinase K (10 mg/ml) (Boehringer Mannheim) and incubation at 37°C for 15 min. Protected fragments were extracted with phenol/chloroform, precipitated with ethanol, resolved by denaturing gel electrophoresis and visualized by autoradiography.

Polysome analysis

5×10^7 cells were washed once in ice-cold Tris–saline solution (25 mM Tris–HCl pH 7.5, 25 mM NaCl) containing 10 mM $MgCl_2$ and 10 μg/ml cycloheximide. The cells were then immediately lysed on ice with the use of a Dounce homogenizer in 2 ml homogenization buffer containing 25 mM Tris–HCl pH 7.5, 25 mM NaCl, 10 mM $MgCl_2$, 2% Triton X-100, 340 U/ml heparin (LEO Laboratories Canada Ltd), 2 mM vanadyl ribonucleoside complex (Sigma), 2.5 mM PMSF, 10 μg/ml cycloheximide, 1 mM dithiothreitol and 1 mM EGTA. The extract was centrifuged at 14 000 r.p.m. for 6 min at 4°C to remove cell debris, the supernatant was collected and layered over a 15–50% linear sucrose gradient (11 ml) prepared in homogenization buffer. The gradients were centrifuged in an SW41 Beckman rotor at 175 000 *g* for 110 min at 4°C. Ten fractions of equal volume were collected from the bottom of the tubes. RNA was prepared from each of the fractions by phenol/chloroform extraction and ethanol precipitation and resuspended in 200 μl DEPC-treated water. The amount of p53 mRNA in each fraction (100 μl of the RNA sample) was determined by dot-blot hybridization analysis using a ^{32}P-labelled human p53 cDNA probe. Polysomes used to calibrate the gradients were prepared in exactly the same way except for an additional purification step involving precipitation of the polysomes present in the homogenate with 100 mM $MgCl_2$ for 1 h on ice before sucrose gradient sedimentation. For calibration, 0.3-ml fractions were collected from the bottom of the gradient and A_{254} of each fraction was determined.

Templates for in vitro transcription and translation

Plasmid p2516 contains nearly full-length human wild-type p53 cDNA and was constructed by the correct ligation of three cDNA fragments. One fragment corresponding to the 5′ end of the p53 transcript was obtained from pR4-2 (Harlow *et al.*, 1985) after digestion with *Xba*I and *Pvu*II which cut in exons 1 and 5, respectively. The middle fragment was obtained from pProSp53 (Matlashewski *et al.*, 1987) after digestion with *Pvu*II and *Bam*HI which cut in exons 5 and 11, respectively. The third fragment corresponding to the 3′ end of the p53 transcript was obtained by RT–PCR amplification of the 3′UTR of p53 mRNA using p53-specific oligonucleotides as primers, 3′SX13 (sense, exon 11, GTCACCCCATCCCACACCCTGG) and AS-4. The PCR-amplified fragment was end-filled with the Klenow fragment of DNA polymerase I and digested at an internal *Bam*HI site. These three fragments which represent contiguous sequences of the native p53 transcript were inserted between the *Xba*I and *Sma*I sites of a modified form of the pSP64 vector (Promega) in which polylinker sequences between the *Hin*dIII site and the *Xba*I site were deleted. The resulting plasmid is referred to as p2516 and yields a p53 transcript *in vitro* starting with the sequence 5′GAATACAAGCTCTAGA.....3′. The *in vitro* transcript is nearly identical to p53 transcripts originating from the most 3′ of the major transcription initiation sites *in vivo* which start with 5′CAAAAGTCTAGA.....3′ (Tuck and Crawford, 1989). The beginning of identity corresponding to an *Xba*I site in the cDNA is underlined. Digestion of p2516 with *Eco*RI provides a template that can produce a synthetic full-length p53 transcript of 2516 nucleotides. Digestion with *Bam*HI provides a template for a truncated p53 transcript of 2034 nucleotides that is missing sequences from the 3′UTR containing the Alu-like element.

The plasmid pCAT-Alu was constructed in two steps. First, the chloramphenicol acetyltransferase gene was excised from the CAT plasmid (Fu *et al.*, 1991) with *Hin*dIII and *Bam*HI, and inserted into pSP64 to generate pSP6CAT. Second, the *Bam*HI–*Eco*RI fragment from p2516 that contains the Alu-like element present in the p53 3′UTR was inserted immediately downstream of the CAT gene. The plasmid pCAT-BS was constructed by removing the *Sma*I–*Bam*HI fragment of the p53 3′UTR present in p2516 and inserting this fragment in reverse orientation into pSP6CAT immediately downstream of CAT. This *Sma*I–*Bam*HI fragment is missing the Alu-like element present at the distal end of the p53 3′UTR.

In vitro transcription and in vitro polyadenylation

Plasmid DNAs containing templates for *in vitro* transcription were linearized at selected restriction endonuclease sites. Standard transcription assays (Melton *et al.*, 1984) were performed as described above for the preparation of antisense RNA probes with the omission of [^{32}P]UTP. 0.5 mM ^{7m}G(5′)ppp(5′)G and 0.05 mM GTP were included in the reactions to provide efficient capping at the 5′ end of synthetic transcripts. Polyadenylation reactions contained synthetic RNA, 0.2 mM ATP, 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 250 mM NaCl, 2 mM $MnCl_2$, 2 mM dithiothreitol, 1 unit/μl RNAguard (Pharmacia), 500 μg/ml of BSA (Pharmacia) and 5 units of poly(A) polymerase (Pharmacia) in a 50 μl final volume (McGrew *et al.*, 1989). After 30 min at 37°C, polyadenylated RNAs were purified by phenol/chloroform extraction and ethanol precipitation.

In vitro translation and immunoprecipitation

Synthetic transcripts were translated in micrococcal-nuclease-treated rabbit reticulocyte lysates (Promega) under the conditions recommended by the supplier. Reactions containing p53 transcripts were incubated for 30 min at 30°C in the presence of [^{35}S]methionine and stopped by addition of dithiothreitol to a final concentration of 1 mM and EDTA pH 8.0 to a final concentration of 10 mM. Each reaction was then divided into two aliquots, one for immunoprecipitation with the p53-specific monoclonal antibody PAb421 and the other for immunoprecipitation with a control antibody PAb419. Reactions containing CAT or luciferase transcripts were incubated for 30 min at 30°C in the presence of [^{35}S]methionine and were stopped by addition of protein sample buffer, boiled for 5 min and resolved by polyacrylamide gel electrophoresis.

Acknowledgements

This work was supported by grants from the Medical Research Council of Canada and from the National Cancer Institute of Canada.

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Received on November 30, 1995; revised on April 18, 1996